REMARKS

The Office Action has maintained the restriction requirement earlier imposed and has withdrawn Claims 27 and 28 from consideration. It has examined Claims 1-26 and 29 and 30 on the merits. The Office Action has rejected Claims 5-26, 29 and 30 under 35 U.S.C. §112, first paragraph, for allegedly being non-enabling. Moreover, Claims 15-25 are rejected under 35 U.S.C. §102(b) as defining subject matter which is allegedly anticipated by the teachings in WO 97/48397, the inventors of which are Biedermann et al. ("Biedermann et al."). Further, Claims 15-25 are rejected under 35 U.S.C. §102(b) as defining subject matter which is allegedly anticipated by an article by Nishikawa et al. in J. Med. Chem. 1989, 32, 583-589 ("Nishikawa et al."). Moreover, Claims 15-26 and 29-30 are rejected under 35 U.S.C. §103(a) as defining subject matter which is allegedly rendered obvious by the teachings in Nishikawa et al. in view of the teachings in an article by Nie et al. in Blood, 2000, 95, 7, 2304-2311 ("Nie et al.") and an article by Colavitti et al. in The Journal of Biological Chemistry 2002, 277, 2, 3101-3108 ("Colavitti et al.").

Applicants have amended the claims which, when considered with the comments hereinbelow, are deemed to place the present application in condition for allowance. Favorable action is respectfully requested.

Before addressing the merits of the Office Action, it is to be noted that claims 15, 25 and 26 have been amended. Claim 15 has been amended to recite a method of treating a disease or medical condition in a mammal which disease or medical condition responds to inhibition or reduction of angiogenesis, listing specific diseases and medical conditions utilizing the compound of Formula I. Support for the amendments to Claim 15 is found on Page 2, Line 28 to Page 3, Line 25 and Page 11, Line 27 to Page 12, Line 24 of the instant specification.

Claim 25 has been amended to change dependency. Support for the amended subject matter in Claim 26 is found on Page 2, Line 28 to Page 3, Line 25 and Page 11, Line 27 to Page 12, Line 24 of the instant specification.

No new matter has been added to the application.

Claims 27 and 28 are withdrawn; applicants reserve the right to file a divisional application directed thereto.

Although Claims 1-14 and Claims 23 and 24 have been cancelled, these claims have been cancelled without prejudice. Applicants have not abandoned the subject matter therein and reserve the right to file a continuation application directed thereto.

Pursuant to the rejection of claims 15-26, 29 and 30 under 35 U.S.C. §112, first paragraph, the Office Action states that the present application is enabling for reducing undesired angiogenesis and thus treating a disease or medical condition selected from rheumatoid arthritis, inflammatory disorder, macular degeneration, psoriasis, retinopathy, preneoplastic lesions and hyperplasia. However, it alleges that the specification does not reasonably provide enablement for the prevention or inhibition of angiogenesis generally, citing Carmeliet et al., stating that on "Page 255, the Authors point out that even if one molecule is blocked, such as VEGF, the cells may switch to another molecule. Thus there is no single pathway to inhibit angiogenesis generally."

Applicants respectfully submit that the claimed subject matter is enabling. Although the claimed subject matter is limited to the specific diseases which the Office Action concurs is enabled, it is respectfully submitted that the present application, as originally drafted and as amended, is enabling in accordance with the requirements of 35 U.S.C. §112, first paragraph.

As indicated in the Office Action, In re Wands, 858 F2d 731, 8 USPQ 2d 1400 (Fed Cir 1988) provides the factors to consider when determining whether a disclosure requires an undue amount of experimentation. However, as shown by the analysis of these factors, hereinbelow, an undue amount of experimentation is not required to practice the present invention. These factors are listed and discussed hereinbelow.

(1) The nature of the invention and the breadth of the Claims

As described hereinabove, the claimed subject matter is directed, inter alia, to a method of treating a disease or medical condition in a mammal which disease or medical condition responds to inhibition or reduction of angiogenesis, wherein the disease or medical condition is selected from rheumatoid arthritis, inflammatory disorder, macular degeneration, psoriasis, retinopathy, preneoplastic lesions and hyperplasia using the compounds of Formula I. In another embodiment, the present invention is directed to, inter alia, a method of treating a disease or medical condition in a mammal which disease or medical condition responds to inhibition or reduction of VEGF production, using the compounds of Formula I wherein the disease or medical condition is the aforementioned diseases or medical conditions. In another embodiment, the present invention is directed, inter alia, to a method of treating or preventing a disease or medical condition which disease or medical condition is the aforementioned disease or medical condition using a compound of Formula I.

As presented, the claims are not broad; they are definite and specific. The claims recite specific diseases, the treatment of which the Office Action has indicated is enabled in the present application. In addition, Formula I, which is administered to the mammal, is defined and the substituents thereon are defined and are limited in scope. The compounds of Formula I does not encompass an infinite number of compounds.

(2) The state of the art and the predictability of the art

There is plenty of art that relates to angiogenesis. Angiogenesis is the term used to describe new blood vessel formation and is a feature of many diseases and physiological conditions. These publications describe that the compounds therein are useful for not only inhibiting angiogenesis but also for treating or preventing angiogenesis.

Attention is directed to U.S. Patent No. 4,599,331 to Schreiber et al, copy enclosed, which provides a class of etianic acid for inhibiting angiogenesis. As another example, attention is directed to U.S. Patent No. 5,135,919 to Folkman et al. which describes fumagillin and its salts having angiogenesis inhibiting activity. These compounds are taught to be useful for treating or preventing angiogenesis. In this vein, attention is directed to the claimed subject matter which is specifically directed to the treatment and prevention of angiogenesis. Other examples, illustrating the credibility of the present utility include U.S. Patent Nos. 5,134,156, 5,693,920 and 5,629,340. Thus, contrary to the allegations in the Office Action, the utility of treating or preventing angiogenesis or the diseases associated therewith is credible to one of ordinary skill in the art.

Moreover, Applicants respectfully submit that the United States Patent and Trademark Office has not met its burden establishing that the application is non-enabling. In order to make a proper rejection, the Office Action has the initial burden to establish a reasonable basis to question the enablement for the claimed invention. In re Wright, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1515 (Fed. Cir. 1993). Case law has held that the specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement

requirement of 35 U.S.C. 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for an enabling support. In re Marzocchi, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971). As stated by the Marzocchi Court, "it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure." 439 F.2d at 224, 169 USPQ at 370.

Here, the Office Action has not provided any rationale supporting its allegation that the full scope of the present invention is not enabled. In particular, the Office Action has not cited any reference which refutes the teaching of the present invention. Moreover, the Office Action has not provided any evidence which disputes the allegation of the utility of the present invention. Thus, the United States Patent and Trademark Office ("USPTO") has not met its burden.

(3) The amount of direction or guidance provided

The instant specification provides specific direction and guidance in practicing the claimed invention, contrary to the allegations in the Office Action. Attention is given to the disclosure on Page 12, Line 18, to Page 13, Line 35 and Page 19, Line 3 to Page 28, Line 16 of the instant specification. The latter text of the specification describes various therapeutic administration forms, including how to administer the compound of Formula I in the various therapeutic forms, while the former text refers to the amount and the dosage regimen to be administered to subjects. Further, attention is given to the text on Page 32, Line 21 to Page 35,

Line 12 of the instant specification, which illustrates using a representative compound of Formula I, the treatment regimen on a test animal.

(4) The presence or absence of working examples

The present specification contains working examples. Attention is directed to the experimental section on Pages 29 et seq. of the instant specification. The first example shows the effect of a representative compound of Formula I on VEGF production in an in vitro model system, HepG2 cells. As shown by Table I on Page 32 and Figure I, the representative compound inhibited the production of HepG2 cells very effectively at concentrations as low as 2 or 20 nm. As shown, after 72 hours, the representative compounds at these low concentrations completely inhibited, within experimental measurements, the production of VEGF in HepG2 cells.

Attention is further directed to the in vivo, experiments described on Page 32 et seq. showing the effectiveness of the administration of a representative compound of Formula I in male Balb/c mice having a RENCA tumor implanted thereon. The drug was given to animals twice a day for six days at various concentrations. As shown by the data in Figures 2 and 3, the representative compound of the present invention, BPPA reduced the vessel density in the RENCA tumor. The data compared the effect of the representative drug to a drug, TNP 470, which was known to be an antiangiogenic compound. As shown, even though TNP-470 was administered at higher concentrations and over longer period of time than BPPA, BPAA was more effective than TNP-470 in reducing the vessel density in the RENCA tumor.

(5) Amount of experimentation

Based on the above, an undue amount of experimentation is not required to practice the present invention. Although some experimentation is permissible, In re Wands, 858

F2d 731, 736-737, 8 USPQ 1400, 1404 (Fed. Cir. 1988), the amount of experimentation in this case is not undue. Further, the USPTO concurs as the diseases recited in the claims have limited to the diseases which the Office Action has considered to be enabled in the specification. Thus, the claimed invention is enabled, and a person of ordinary skill in the art would not have to engage in an undue experimentation in practicing the present invention.

Therefore, for the reasons given herein, the rejection of the claims under 35 U.S.C. §112, first paragraph is obviated; withdrawal thereof is respectfully requested.

Pursuant to the rejection of Claims 15-25 under 35 U.S.C. §102(b), the Office Action cites Biedermann et al.

Biedermann et al. disclose the use of compounds of Formula I therein for the treatment of tumors or for immunosuppression. There is no teaching or disclosure therein of the use of the compounds therein for the treatment of rheumatoid arthritis, inflammatory disorder, macular degeneration, psoriasis, retinopathy, preneoplastic lesions, and hyperplasia.

Case law has held that a claim is anticipated if the prior art reference teach and disclose every element of the claims, either explicitly or implicitly. MGHL/Biophile Int'l Corp. v. Milgram, 192 F3d 1362, 1365, 52 USPQ2d 1303, 1305 (Fed. Cir. 1999). The absence in the prior art of any element in the claims negates anticipation. Kalman v. Kimberly Clark Corp., 713 F2d 760, 771-771, 218 USPQ 781, 789 (Fed. Cir. 1988).

Inasmuch as Biedermann et al. do not teach or disclose the treatment of the specific diseases enumerated in Claims 15-25, Biedermann et al. do not disclose every element in the claims. Consequently, Biedermann et al. do not anticipate the subject matter of Claims 15-25. Therefore, this rejection is obviated; withdrawal thereof is respectfully requested.

Pursuant to the rejection of Claims 15-25 under 35 U.S.C. §102(b), the Office Action cites Nishikawa et al. According to the Office Action, Nishikawa et al. describes the administration of compounds 18a, having anti-allergic activity.

A review of Nishikawa et al. clearly reveals that Nishikawa et al. do not teach or disclose any of the diseases recited in the claimed subject matter. Thus, this rejection under 35 U.S.C. §102(b) is obviated; withdrawal thereof is respectfully requested.

Pursuant to the rejection of Claims 15-26 and 29-30, the Office Action cites Nishikawa et al. in view of Die et al. and Colavitti et al.

As described herein, Nishikawa et al. describe the use of Compound 18, i.e., [4-(1-benzyl-piperidin-4-yl)-butyl]-3-(pyridin-3-yl)propionamide as an antiallergic agent. The Office Action alleges that this compound is a lipoxygenase inhibitor. The Office Action cites Nie et al. for alleging that it teaches that lipoxygenase inhibitors can counteract the effects of VEGF, and it cites Colavitti et al. for the proposition that VEGF is a major angiogenic factor.

Upon a closer review, it appears that USPTO has overgeneralized. Nishikawa et al. disclose that the compounds therein are useful for inhibiting 5- lipoxygenase, while Nie et al. disclose that 12- lipoxygenase inhibitors inhibit angiogenesis. As shown in Nie et al, there are various lipoxygenases, such as 5-LOX, 12-LOX and 15-LOX and there is no teaching in either reference that 5-LOX can be substituted for 12-LOX..

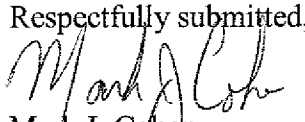
Colavitti et al. are being cited to identify VEGF as the major anaiogenic factor, and does not address the issue. There is no teaching or suggestion in the reference that a 5-lipoxygenase inhibitor can substitute for a 12- lipoxygenase or vice versa or that they both inhibit angiogenesis. Since chemistry is an unpredictable, there is no expectation, without more, that a compound, which is a 5- lipoxygenase inhibitor, can inhibit angiogenesis.

In evaluating obviousness, it is axiomatic to look "to see if the prior art would have suggested to one of ordinary skill in the art that process this should be carried out and would have a reasonable likelihood of success, reviewed in light of the prior art." In re Dow Chemical Company, 837 F2d 469, 473 (Fed. Cir. 1988). Here, there is no teaching or suggestion in any of the references that the compound disclosed in Nishikawa et al. would be useful as an inhibitor of angiogenesis, or that it would be useful for treating (or preventing) any of the diseases recited in the claims. Accordingly, the Office Action has not made out a prima facie case of obviousness.

Accordingly, this rejection is obviated and withdrawal thereof is respectfully requested.

In view of the Amendment to the claims and the arguments herein, it is respectfully submitted that the present application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,



Mark J. Cohen
Registration No. 32,211

Scully, Scott, Murphy & Presser, P.C.
400 Garden City Plaza – Suite 300
Garden City, New York 11530
(516) 742-4343

MJC:htj

United States Patent [19]

Schreiber et al.

[11] **Patent Number: 4,599,331**

[45] **Date of Patent: Jul. 8, 1986**

[54] **ETIANIC ACIDS AS ANTIANGIOGENICS**

[75] Inventors: **Alain B. Schreiber**, Sunnyvale; **William J. Kowalski**; **Stefan H. Unger**, both of Palo Alto, all of Calif.

[73] Assignee: **Syntex (U.S.A.) Inc.**, Palo Alto, Calif.

[21] Appl. No.: **685,568**

[22] Filed: **Dec. 24, 1984**

[51] **Int. Cl.**[4] **A61K 31/56**

[52] **U.S. Cl.** **514/179**; 260/397.1

[58] **Field of Search** 260/397.1; 514/179

[56] **References Cited**

U.S. PATENT DOCUMENTS

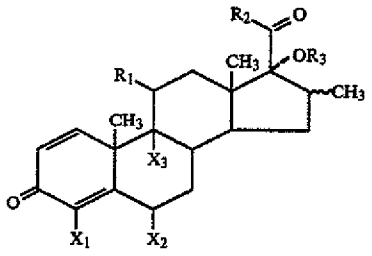
4,198,403	4/1980	Alvarez	260/397.1
4,310,466	1/1982	Edwards	260/397.1

Primary Examiner—Elbert L. Roberts

Attorney, Agent, or Firm—Charles L. Hartman; Tom M. Moran; Alan M. Krubiner

[57] **ABSTRACT**

This invention provides a method for inhibiting angiogenesis in mammals. A pharmaceutically acceptable formulation containing a compound of the formula



where X_1 X_2 and X_3 are H, Cl, or F and X_3 must be H if the substituent of R_1 is αOH;

R^1 is chosen from the group consisting of hydroxyl and hydrogen;

R_2 is chosen from the group of alkyloxy or thioalkyls wherein said alkyls have less than 5 carbon atoms

R_3 is H or an alkyl having less than 6 carbon atoms, is administered to the mammal having undesirable angiogenesis.

The wavy line at C-16 refers to either α or β.

10 Claims, No Drawings

ETIANIC ACIDS AS ANTIANGIOGENICS

BACKGROUND OF THE INVENTION

This invention relates to the use of novel steroid compounds, particularly to the use of compounds of the class of etianic acids, for inhibition of blood vessel formation. Certain etianic acids and esters derived therefrom can inhibit blood vessel formation, and can be used in treating solid tumor growth, ophthalmic retinopathies including diabetic, and granulomatous disease, and as a contraceptive, as well.

"Angiogenesis" is the term used to describe new blood vessel formation. Angiogenesis is a feature of many diseases and physiological conditions, for example, ophthalmic retinopathies, granulomatous disease, and solid tumor growth, as well as wound healing and corpus luteum formation. Folkman discusses angiogenesis in a general way in *Ann. N.Y. Acad. Sci.* 1982, p. 212.

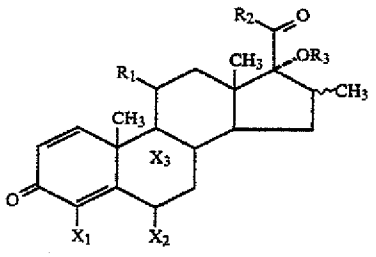
Conditions characterized by angiogenesis may be treated by inhibiting angiogenesis, if the treatment has few side effects. For example, angiogenesis accompanies most solid tumor growth. The prevention of further angiogenesis can inhibit further tumor growth in some cases. As another example, preventing angiogenesis is an effective means of contraception in mammals.

Folkman has demonstrated both that angiogenesis is necessary for tumor growth, and that tumor growth stops if angiogenesis is inhibited. See *Science*, 221, Aug. 1983, p. 719–725. He reported that if angiogenesis was inhibited by a combination of heparin, or heparin fragments, and cortisone, tumor growth could be stopped and even reversed. He always administered both heparin and a glucocorticoid steroid to achieve the anti-angiogenic effect. One disadvantage that he has recognized is that not all heparins give identical results. (See *Science* 221 at p. 722) And, in one case, the heparin that gave the best anti-angiogenic activity was removed from the market during the pendancy of the study. Later, he reported the successful use of hydrocortisone and heparin to inhibit angiogenesis in *The Third International Symposium on the Biology of Vascular Endothelial Cell*, at Cambridge, Mass. June 25–29, 1984.

It would be advantageous to find a class of steroid compounds that are anti-angiogenic, that do not have any other biological effects, particularly severe glucocorticoid effects. The applicant has found that etianic acids and their esters do show anti-angiogenic effects.

SUMMARY OF THE INVENTION

This invention provides a method for inhibiting angiogenesis in mammals. A pharmaceutically acceptable formulation containing a compound of the formula



FORMULA I

where X_1 X_2 and X_3 are H, Cl, or F,

where X_3 must be H if the substituent of R_1 is αOH;

R_1 is chosen from the group consisting of hydroxyl or hydrogen

R_2 is chosen from the group of alkyloxy or thioalkyls wherein said alkyls have less than 5 carbon atoms;

R_3 is H or an alkyl having less than 6 carbon atoms, is administered to the animal having undesirable angiogenesis.

The wavy line at C-16 refers to either α or β.

"Inhibiting angiogenesis" as used in the context of this invention means preventing further vascular development or slowing further development of vascularization. The longer term use of the anti-angiogenic etianic esters of this invention can result in the destruction of vessels due to angiogenesis already present, and this phenomenon is also included in the term.

Unless a specific formula is intended, the term "etianic acid ester" as used herein will refer to the generic class of compounds of Formula 1. Examples of such etianic acid esters are shown in U.S. Pat. Nos. 4,198,336, 4,278,699, 4,261,986, 4,198,404, 4,198,403, 4,261,984, 4,187,301, 4,188,385 and 4,263,289.

DETAILED DESCRIPTION

As an untreated tumor grows, blood vessels in and around it will typically proliferate. One can easily visually determine which blood vessels are due to tumor related angiogenesis, because these vessels are particularly tortuous, and, in advanced cases, are characterized by localized blood leakage and hemorrhage. The administration of etianic acids inhibits the vascularization that normally accompanies tumor growth and as a result, the tumor fails to grow. If its growth is prevented long enough, the tumor may regress.

Other diseases, for example ophthalmic retinopathies and granulomatous diseases can be treated similarly. Etianic esters are useful as contraceptives, since they can prevent formation of the corpus luteum required for embryonal development.

Effective systemic administration includes oral administration, and subcutaneous or intraperitoneal injection. The present invention is also useful for those localized diseases, such as skin tumors and the like, where topical administration is possible.

Depending on the intended mode of administration, the compositions used may be in the form of solid, semi-solid or liquid dosage forms, such as, for example, tablets, suppositories, pills, capsules, powders, liquids, suspensions, or the like, preferably in unit dosage forms suitable for single administration of precise dosages. The compositions will include a conventional pharmaceutical carrier or excipient and an active compound of Formula I and, in addition, may include other medicinal agents, pharmaceutical agents, carriers, adjuvants, and the like.

For solid compositions, conventional non-toxic solid carriers that may be used include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. Liquid pharmaceutically administerable compositions can, for example, be prepared by dissolving, dispersing, etc. an active compound as defined above and optional pharmaceutical adjuvants in a carrier, such as, for example, water, saline, aqueous dextrose, glycerol, ethanol, and the like, to thereby form a solution or suspension. If desired, the pharmaceutical composition to be administered may also contain minor amounts of nontoxic auxiliary substances such as wetting or emulsifying agents,

pH buffering agents and the like, for example, sodium acetate, sorbitan monolaurate, triethanolamine sodium acetate, triethanolamine oleate, etc. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pa., 15th Edition, 1975. The composition or formulation to be administered will, in any event, contain a quantity of the active compound(s) in an amount effective to alleviate the symptoms of the subject being treated.

For the compounds of formula I, either oral or topical administration is preferred depending on the nature of the disorder being treated.

For oral administration, a pharmaceutically acceptable non-toxic composition is formed by the incorporation of any of the normally employed excipients, such as, for example pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium, carbonate, and the like. Such compositions take the form of solutions, suspensions, tablets, pills, capsules, powders, sustained release formulations and the like. Such compositions may contain 1%–95% active ingredient, preferably 25–70%.

For topical administration, these compositions comprise an effective amount of a compound of this class in a mixture with a pharmaceutically acceptable non-toxic carrier. A suitable range of composition would be 0.1% – 10% active ingredient, and the balance carrier, preferably 1–2% active ingredient.

Some members of the class of etianic acids are known to have glucocorticoid effects, but there is no relationship between anti-angiogenic activity and glucocorticoid activity. Some compounds known to be powerful glucocorticoids have little or no anti-angiogenic activity and some compounds known to have no glucocorticoid activity have anti-angiogenic activity. All of the etianic esters, as defined above, have at least some anti-angiogenic activity.

The ideal compound would have high anti-angiogenic efficiency, but no glucocorticoid, or other steroid or other undesirable side effects. To screen the series of etianic esters each was tested for glucocorticoid activity using a binding competition test, the tyrosine amino transferase induction test, and the alkaline phosphatase induction test.

Each compound of the series was also tested for anti-angiogenic activity by an in vivo test that measured vascularization in a hamster's cheek pouch in response to either an angiogenic polypeptide growth factor, epidermal growth factor (EGF) or an induced timer. The results of all the tests, for the ester and thio ester subclasses of etianic esters series, are tabulated in Tables 1, 2, and 3.

EXAMPLE 1

In this example, the affinities of test compounds for the glucocorticoid receptor were determined by competition for ^{3}H-dexamethasone (DEX) binding sites in cytosols from tissue culture cells.

LE II (mouse lung capillary endothelial) or HTC (rat hepatoma) cells were grown in Dulbecco's modified Eagle's (DMEM) medium plus 10% fetal calf serum (FCS), in 150 cm^2 T-flasks. Approximately 20 flasks of LE II cells or 10 flasks of HTC cells were needed for a 60–80 sample assay.

Cell monolayers were washed 3 times with Phosphate Buffered Saline (PBS) and harvested by scraping with a rubber policeman into an ice-cold sonication buffer of 0.25M sucrose; 10 mM Tris-HCl, pH 7.4; 5 mM ethylenediaminetetraacetic acid (EDTA); 5 mM dithiothereitol; and 10 mM sodium molybdate. All subsequent steps were performed at 4° C. The cells were pooled into a final volume of 8–12 mls, broken by sonication for 45 seconds with the microtip of a Branson sonifier at setting #3 and the residue was then centrifuged at 100,000 X g for 30 minutes with a Beckman L8-50, Ti50 rotor, at 40,000 rpm. Individual portions of the cytosol were incubated with 25 nM ^{3}H-DEX, either with or without unlabeled test compounds, at concentrations between 0.25–250 nM. Incubations were peformed in a final volume of 1 ml in 1.5 ml Eppendorf centrifuge tubes for 3 hours on ice.

Separation of free from protein-bound ^{3}H-DEX was accomplished by chromatography on Sephadex G-50 minicolumns. Fine Sephadex G-50 gel was swollen and equilibrated against column buffer of 100 mM Tris-HCl, pH 7.8; and 3 mM $CaCl_2$. Columns were poured to a final packed volume of 4.5 ml in 5 ml plastic disposable pipets.

The packed columns were then drained of buffer and the 1 ml incubated samples were applied to each. The samples were washed into the columns with 0.5 ml of column buffer, and the exclusion volumes containing the protein peaks were eluted with another 1 ml of column buffer and collected. Aliquots of 750 μl of Biorad protein reagent were diluted 1:5 in H_2O. OD_{630nm} reading were compared with those obtained for a bovine serum albumin standard curve. See the BHTC and BLE11 Columns in Tables 1 and 2.

Final values are expressed as fmoles ^{3}H-DEX bound per mg of protein. Scatchard analyses show that LE II cells bind approximately 500 fmoles of ^{3}H-DEX per mg at saturation, with an equilibrium dissociation constant of 8×10^{-9}M; while HTC cells bind about 1100 fmoles of ^{3}H-DEX per mg protein at saturation, with an equilibrium dissociation constant of 4×10^{-9}M.

Results are expressed in Tables 1 and 2 as the concentration required for 50% inhibition $(IC)_{50}$ in displacement experiments.

EXAMPLE 2

In this example, tyrosine aminotransferase (TAT) activity was determined by a spectrophotometric assay. This enzyme catalyzes the conversion of tyrosine to p-hydroxyphenylpyruvate. This example is a standard in vitro test for glucocorticoid activity.

Addition of base caused the conversion of p-hydroxyphenylpyruvate to p-hydroxybenzaldehyde, which was quantitatively measured by absorbance at 331 nm. HTC cells, the target cells for the compound tested were grown in DMEM + 10% FCS. Subconfluent cell cultures were incubated with test compounds in 6 well Costar dishes for 24 or 48 hours. The cells were incubated in either serum-free DMEM or DMEM containing 10% FCS previously filtered over activated charcoal. The cells were harvested in PBS using a rubber policeman, then washed once in PBS by centrifugation and resuspended in a 0.3 ml lysis buffer of 10 mM Tris, 10 mM EDTA and 0.25M sucrose at pH 7.4. The cells were then lysed by three cycles of freeze-thawing using dry ice and a 37° C. waterbath and the cells debris was pelleted by centrifugation for 2 min at 12,000 9 in an Eppendorf centrifuge.

The supernatant was used for a protein determination using either E+K stain or BioRad protein assay kit and TAT activity. 0.1 ml TAT buffer having 50 mM KH_2PO_4, 1 mM EDTA, 0.1 mM Dithiothereitol (DTT), and 5 mg/ml BSA at pH 7.6 and 0.1 ml cell extract were mixed in glass tubes. A prewarmed mixture of 200 parts of a 2.3 mg/ml tyrosine solution in 0.05M phosphate buffer, 100 parts 0.125M KH_2PO_4, 10 parts of a 0.92 mg/ml and ketoglutarate solution and 1 part of a 0.04 mg/ml pyridoxalphosphate solution had been previously made up. 9.7 ml of the prewarmed mixture was added to each tube and allowed to react at 37° C. for 15 min. The addition of 0.1 ml 7N HOH stopped the reaction. The mixture was then immediately vortexed.

Aldehyde formation was allowed to proceed at 37° C. for 30 min. The amound of aldehyde formed was determined by UV spectroscopic means. Absorbance was read at 331 nm using a solution composed of 0.1 ml TAT buffer, 0.1 ml lysis buffer, 0.7 ml reaction mixture and 0.1 ml 7N KOH as a blank. Optical Density values are linearily corrected for protein content.

As the absolute amount of enzyme induction varies between experiments, results are expressed as percent of the maximal induction obtained with dexamethasone, which was run as a standard in each assay. Dexamethasone induces maximal TAT activity at 10^{-7}M with an EC_{50} ~2×10^{-8}M.

In this series, compounds were assayed from 10^{-6}M down. Results are expressed in the TAT column of tables 1, and 2 as the induction ability of a compound as compared to dexamethasone; a compound may either be a partial inducer or a superinducer.

EXAMPLE 3

This example shows a test for alkaline phosphatase (APb) activity. This is another standard test for glucocorticoid activity.

LE II cells were grown in DMEM plus 10% FCS, at 37° C., in a 10% carbon dioxide atmosphere. Confluent monolayers of cells were trypsinized and seeded at a 1:15 dilution into 6-well Costar cluster dishes. After allowing 24 hours for attachment, the culture medium was replaced with fresh medium, either with or without the test compound. In all experiments, DEX, at concentrations of 10^{-9} to 10^{-6}M, was added to some of the wells as a positive control. Preliminary experiments had shown that maximum induction of alkaline phosphatase activity by DEX was seen after 48–72 hours of incubation; for routine experiments, the cells were incubated for about 65 hours.

Cell monolayers were washed with PBS and harvested by scraping with a rubber policeman into 400 µl of a buffer of 20 mM Tris-HCl at pH 7.4; 2 mM $MgCl_2$; and 150 mM NaCl together with 1% Triton X-100. Samples were transferred into 1.5 ml Eppendorf centrifuge tubes and placed on ice. The tubes were first vortexed and then centrifuged in the Eppendorf minicentrifuge for 2 minutes. Duplicate 100 µl aliquots of supernatant were placed into 12×75 mm glass test tubes for determination of APb activity, while the rest was saved for protein determination. The alkaline phosphatase reaction was initiated by the addition to the tubes of 0.5 ml of 10 mM p-nitrophenylphosphate (Sigma) in 0.1M ethanolamine, pH 10.5. The tubes were incubated in a 37° C. water bath for between 20 to 50 minutes, depending on the activity of the samples. The APb reaction was terminated by the addition of 0.5 ml of 0.2 NaOH. The tubes were mixed and 200 µl portions from each tube were transferred in triplicate to a 96 well microtiter plate. The OD_{410}nm of each well was then determined using a Dynatech plate reader. The micromoles of product formed were determined from the molar extinction coefficient of nitrophenol at 410 nm.

Triplicate µl aliquots of the cell supernatant were transferred to 96 well microtiter plates for protein determination by a modification of the Lowry method. Fifty µl of a reaction mixture containing 0.19M Na_2CO_3; 0.1N NaOH; 0.7 mM sodium-potassium tartrate; and 0.8 mM $CuSO_4$ were added to each well, followed by the addition of 50 µl of 10% sodium dodecyl sulfate-5% Folin-Ciocalteu reagent (Sigma). The plates were shaken for 1 hour and then the OD_{630} was determined using a Dynatech plate reader. The protein content for each sample was calculated by comparing the average OD_{630} reading with those obtained for a bovine serum albumin standard curve.

The alkaline phosphatase activity of in each sample was expressed as µmoles product formed per minute per mg protein in the Alk P column of Tables 1 and 2. Results are expressed as the induction ability of a compound as compared to dexamethasone.

EXAMPLE 4

In this example, the in vivo angiogenesis inhibition activity of etianic acid esters was tested. Male Syrian Golden hamsters, weighing between 120 g and 150 g were anaesthetized by intraperitoneal (I.P.) injection of a rodent anaesthetic mixture consisting of 50 mg/kg ketamine hydrochloride, 5 mg/kg xylazine and 1 mg/kg acepromazine. The left cheek pouch of each animal was everted with a pincet and pinned down to a plexiglass-rubber stage and observed through a Zeiss stereomicroscope. 10 µg of EGF or 3×10^5 Hamster Melanoma RPM1 1846 tumor cells in 10 µl Cibachrome Blue agarose (Amicon) were injected subcutaneously in the left lower quadrant of the pouch with a Hamilton syringe.

The pouch was then reinserted and animals were kept under observation until recovered from anaesthesia.

The animals were dosed daily with one test compound, starting one day before stimulus injection and continuing until observation. The test compounds were dissolved in a minimum volume of ethanol and diluted in sterile phosphate buffer solution. Then between 0.5 and 4 mg/kg of the test compound was injected subcutaneously.

Either five days or twelve days after the EGF or tumor cell injection, animals were anaesthetized by I.P. injection of the rodent anaesthetic mixture. The pouch was everted on the stage and examined under the stereomicroscope. Pictures were taken with a 35 mm using Kodak Tungsten 50 professional films.

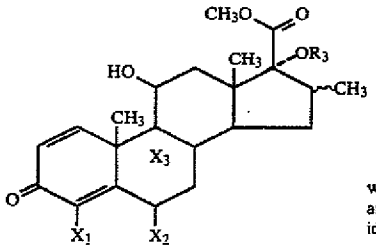
The following subjective scoring was used for evaluating the photographs:

0: no new vessels.
1: minimal branching of vessels in the vicinity of injection site.
2: new vessels reach injection site, area involved is minimal (<50% of area surrounding injection site).
3: many vessels reach and cross injection site, new vessels are tortuous, (>50% of area surrounding injection site).
4: "full blown", many tortuous vessels, leakages, hemorrhages, 100% of area around injection site, invasion of new vessels into non-injected areas.

In the case of the tumor, tumor size was also determined. (See Table 4)

Results are expressed as the mean of the score. The results are shown in Tables 1 and 2 are percent inhibition of HCP Stimulus. Results of a two week assay for various compounds are shown in Table 3.

TABLE 1



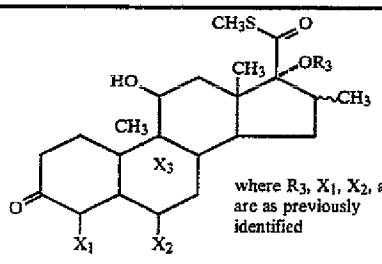
where R_3, X_1, X_2, and X_3 are as previously identified

	X_1	X_2	X_3	16	R_3	IC_{50} (nM) Binding HTC	IC_{50} (nM) Binding LEH	Efficacy (DEX = 1) TAT	Efficacy (DEX = 1) Alk P	% Inhibition HCP Stimulus Epidermal Growth Factor	% Inhibition HCP Stimulus 1846
1	H	H	F	β	PROP	5	2	1.0	1.0	70, 77	40
2	H	F	F	β	PROP			0.8	0.9	100, 100	33, 35
3	F	F	F	β	PROP	15	15	1.5	1.0	95, 91	70, 70
4	H	F	F	β	BUT	0.25	0.25	1.0	1.7	100, 78	26
5	H	F	F	α	FORM					--25	
6	H	F	F	α	ACET			0.7		23	
7	H	F	F	α	PROP	4	0.25	2.0	1.1	57	
8	F	F	F	α	PROP	0.25	0.25	1.0	1.2	41, 58, 100, 72, 82	75
9	H	F	F	α	BUT	0.025	0.025	0.9	0.9	100	32
10	H	F	F	α	VAL	0.025	0.025	0.9	0.7	100	24

Each tabulate number is the result of tests with six animals. These show independent results, each the average of six animals.

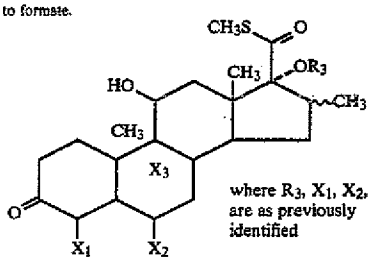
In this table PROP refers to propionate, BUT to butyrate, VAL to valerate, and FORM refers to formate.

TABLE 2



where R_3, X_1, X_2, and X_3 are as previously identified

	X_1	X_2	X_3	16	R_3	Efficacy (DEX = 1) TAT	HCP[a] % Inhibition Stimulus EGF	HCP[a] % Inhibition Stimulus 1846
11	H	F	F	β	PROP	1.4	100	28



where R_3, X_1, X_2, and X_3 are as previously identified

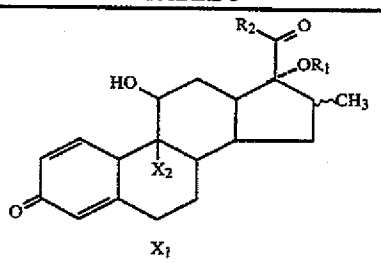
TABLE 2-continued

	X_1	X_2	X_3	16	R_3	Efficacy (DEX = 1) TAT	HCP[a] % Inhibition Stimulus EGF	HCP[a] % Inhibition Stimulus 1846
12	H	H	F	β	BUT	1.3	37	
13	H	F	F	β	BUT	0.9	94, 84	40, 53, 45
14	H	F	F	β	VAL	0.6	22, 42	16
15	H	H	F	α	PROP		30	
16	H	F	F	α	PROP	1.2	46	25, 35
17	F	F	F	α	PROP			
18	H	F	F	α	BUT	1.3		16

[a]Compounds were given daily at 0.4 mg/kg.

In this table PROP refers to propionate, BUT to butyrate, and VAL to valerate.

TABLE 3

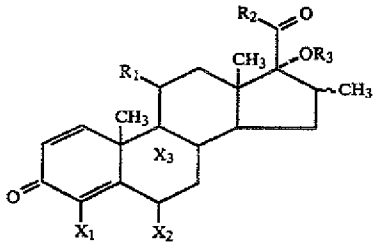


Inhibition of tumor growth (2 week assay)

No.	X_1	X_2	C_{16}	R_1	R_2	dose (mg/kg)	Angiogenesis score	Angiogenesis % Inh.	Tumor area Mn (mm^2)	Tumor area %
[A]	None						3.1	0	40.7 ± 16.2	0
13	F	F	β	butyrate	SCH_3	0.8	1.8	42	9.1 ± 3	78
9	F	F	α	butyrate	OCH_3	0.8	2.2	29	5.7 ± 5.9	86
10	F	F	α	valerate	OCH_3	0.8	2.4	23	22.5 ± 13.7	45
[B]	None						2.9	0	27.5 ± 15.3	0
2	F	F	β	propionate	OCH_3	0.8	1.9	35	11.6 ± 10.3	58

What is claimed is:

1. A method for inhibiting angiogenesis in animals comprising administering a pharmaceutically acceptable formulation containing a compound of the formula



where X_1, X_2 and X_3 can be the same or different and can be hydrogen, fluorine and chlorine;

R_1 is chosen from the group consisting of hydrogen and hydroxyl;

R_2 is chosen from the group consisting hydrogen, methoxy and thiomethyl; and

R_3 is chosen from the group consisting of alkyl groups having less than 6 carbon atoms.

2. The method of claim **1** wherein said compound includes those compounds having R_1 as hydroxyl, and R_2 as methoxy.

3. The method of claim **2** wherein said compound includes those compounds having R_3 as propionate.

4. The method of claim **2** wherein said compound includes those compounds having X_1 as hydrogen, and X_2 and X_3 are fluorine.

5. The method of claim **3** wherein said compound includes 16 β methyl and 11 β hydroxy.

6. The method of claim **2** wherein said compounds include those compounds having R_3 as buterate.

7. The method of claim **2** wherein said compound includes those compounds having X_1, X_2, and X_3 as fluorine.

8. The method of claim **1** wherein said angiogenesis is inhibited by the propionic ester of 4, 6α, 9α-trifluoro-11β, 17α-hydroxy-16β-methyl 3-oxoandrosta-1,4-diene-17β carboxylic acid.

9. A method for treating granulomatous disease in mammals comprising administering a therapeutically effective amount of the compound of claim **1**.

10. A method of contraception in mammals comprising administering a therapeutically effective amount of the compound of claim **1**.

* * * * *

US005135919A

United States Patent [19]

Folkman et al.

[11] Patent Number: 5,135,919

[45] Date of Patent: Aug. 4, 1992

[54] METHOD AND A PHARMACEUTICAL COMPOSITION FOR THE INHIBITION OF ANGIOGENESIS

[75] Inventors: **Judah Folkman**, Brookline, Mass.; **Takeshi Fujita**, Takarazuka, Japan; **Donald Ingber**, Boston, Mass.; **Tsuneo Kanamaru**, Takatsuki, Japan

[73] Assignees: **Children's Medical Center Corporation**, Boston, Mass.; **Takeda Chemical Industries, Ltd.**, Japan

[21] Appl. No.: **173,305**

[22] Filed: **Mar. 25, 1988**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 145,407, Jan. 19, 1988, abandoned.

[51] **Int. Cl.**[5] **A61K 31/715**; A61K 31/70

[52] **U.S. Cl.** **514/56**; 514/24; 514/58; 514/475

[58] **Field of Search** 514/24, 56, 58, 475; 536/103

[56] **References Cited**

U.S. PATENT DOCUMENTS

2,803,586	8/1957	Peterson et al.	435/244
2,844,512	7/1958	Eble	514/24
3,232,833	2/1966	Riviere	514/54
4,727,064	2/1988	Pitha	536/103

OTHER PUBLICATIONS

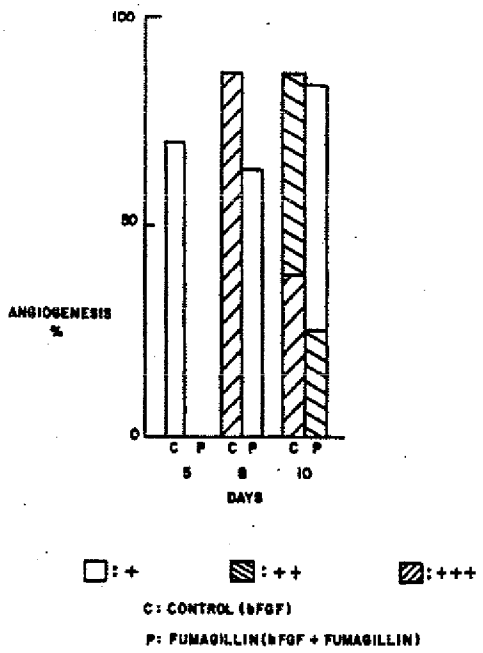
Maevskii et al., *Chemical Abstracts*, 70:36365g (1969).
Semenov. et al., *Chemical Abstracts*, 61:1709h (1964).
R. Crum, *Science*, vol. 230, p. 1375 (1985).
Folkman, et al. Science 230:1375–1378 (1985).
Folkman, et al., Journal of Cell Biology, vol. 107, No. 6, part 3, p. 579a, Abstract No. 3277 (1988).
Taylor, et al., Nature, 297:307–312 (1982).
Tursunkhodzhaev. Chemical Abstracts, 63:2251e (1965).
J. Folkman; Advances in Cancer Research vol. 43, pp. 175–203 1985 ed. George Klein and Sidney Weinhouse.
Proc. Nat. Acad. Sci. USA, 48, 733–735 (1962).
Folkman, J. et al., J. Exp. Med., 133 275 (1971).
Kimura, M. et al., J. Pharmacobiol. Dyn., 9, 442 (1986).
Gimbrone, M. A. J. et al., J. Natl. Cancer Inst. 52, 413 (1974).
DiPaolo, et al. (Carcinolytic Activity of Fumagillin) Antibiotics Annual; 541–546 (1958–1959).
The Merck Index Tenth Edition; 611–612 (1983).

Primary Examiner—Ronald W. Griffin
Assistant Examiner—Pamela S. Webber
Attorney, Agent, or Firm—Gregory D. Williams; David G. Conlin; David R. Resnick

[57] **ABSTRACT**

Fumagillin and its salts have an angiogenesis inhibiting activity and are useful for prophylaxis and treatment of diseases induced by abnormally stimulated neovascularization. The invention also provides certain pharmaceutical compositions comprising fumagillin or its salt, and an agent which potentiates angiogenesis inhibition such as heparin and sulfated cyclodextrins such as beta-cyclodextrin tetradecasulfate.

13 Claims, 1 Drawing Sheet



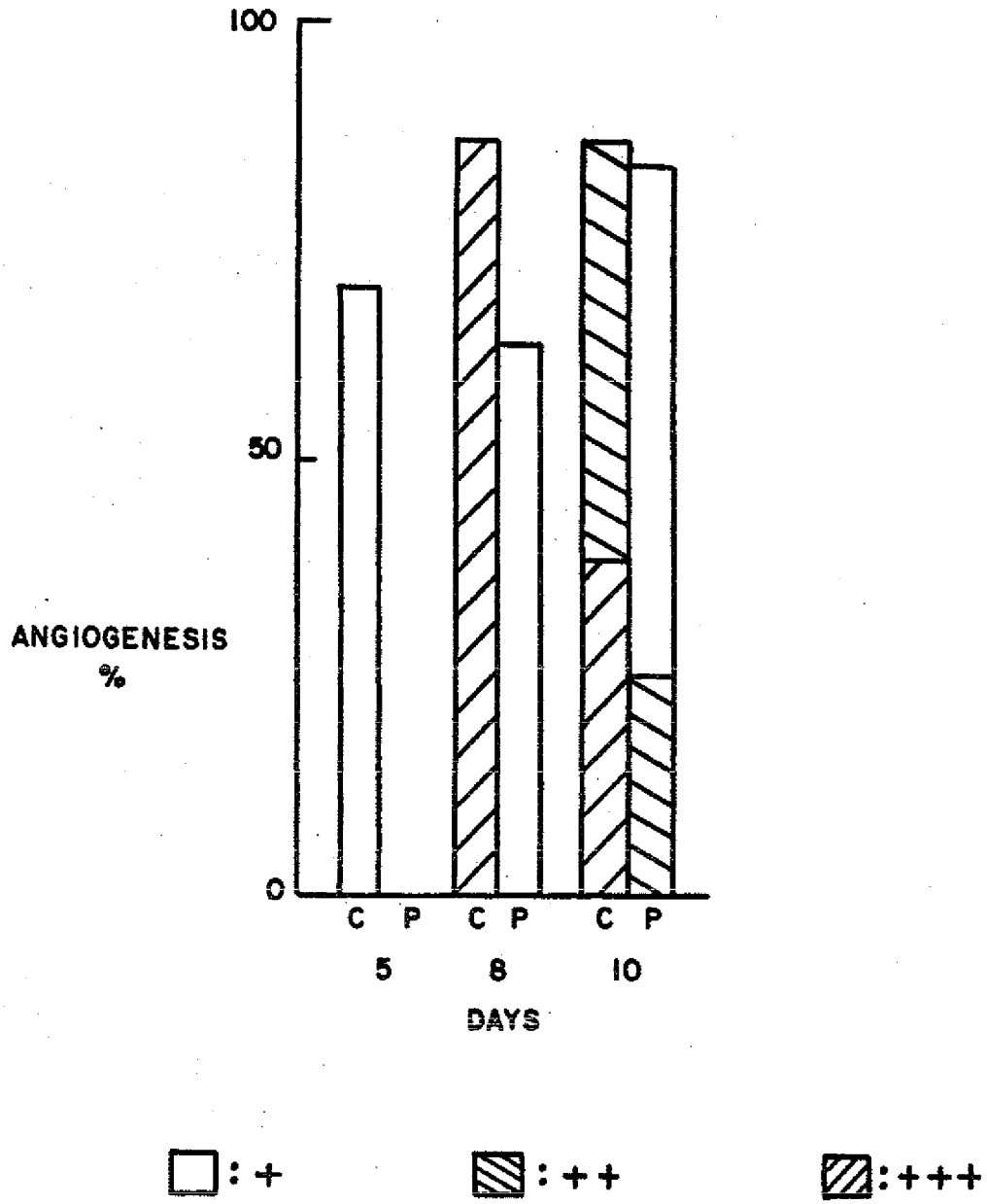

100
50
0
ANGIOGENESIS
%
C P C P C P
5 8 10
DAYS
□ : +
▨ : ++
▨ : +++
C : CONTROL (bFGF)
P : FUMAGILLIN (bFGF + FUMAGILLIN)

FIG. 1

METHOD AND A PHARMACEUTICAL COMPOSITION FOR THE INHIBITION OF ANGIOGENESIS

This is a continuation-in-part application of U.S. Ser. No. 145,407, filed Jan. 19, 1988, now abandoned.

The present invention relates to methods of prophylaxis or treatment of angiogenesis-related diseases induced by abnormally stimulated neovascularization in mammals. The invention also relates to certain pharmaceutical compositions comprising fumagillin and potentiators of angiogenesis inhibition.

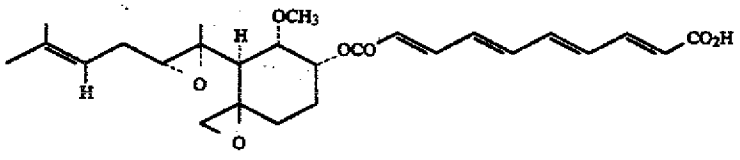
BACKGROUND OF THE INVENTION

Angiogenesis is a process by which new capillary blood vessels are formed. This process occurs normally, e.g., during ovulation and formation of the placenta. It also occurs pathologically in wound healing, as well as in a variety of diseases where uncontrolled or rampant capillary growth is the cause of extensive tissue damage. Examples of the latter are in ophthalmology: diabetic retinopathy, retrolental fibroplasia, corneal graft neovascularization, neovascular glaucoma, ocular tumors and trachoma, where neovascularization may lead to blindness; in dermatology: psoriasis and pyogenic granuloma; in pediatrics: hemangioma, angiofibroma, and hemophiliac joints; in surgery: hypertrophic scars, wound granulation and vascular adhesions; in internal medicine: rheumatoid arthritis, where new vessels in the joint may destroy articular cartilage and scleroderma; in cardiology: atherosclerotic plaque; and in cancer: many kinds of carcinomas and sarcomas, where progressive growth is dependent upon the continuous induction of angiogenesis by these tumor cells.

The realization that tumors, as well as many non-neoplastic diseases, are angiogenesis-dependent has led to a search for angiogenesis inhibitors that might be used therapeutically (See e.g., J. Folkman; Advances in Cancer Research Vol. 43, pp 175–203, 1985 ed. George Klein and Sidney Weinhouse).

Fumagillin is a known compound which has been used as an antimicrobial and antiprotozoal. Its physicochemical properties and method of production are well documented [Production; U.S. Pat. No. 2,803,586: Structure; Proc. Nat. Acad. Sci. USA, 48, 733–735 (1962)].

Fumagillin has a molecular formula of $C_{26}H_{34}O_7$. Its molecular weight is 458.53. Yellow needles from methanol extraction have an mp of about 194°–195° C. Fumagillin has the following structure:



SUMMARY OF THE INVENTION

The present invention relates to the use of the fumagillin, obtainable from *Aspergillus fumigatus*, as an angiogenesis inhibitor. Fumagillin shows strong angiogenesis inhibitory activity and may be used to treat a variety of diseases induced by abnormally stimulated angiogenesis including diabetic retinopathy, trachoma, and the like. The present invention also relates to certain pharmaceutical compositions comprising fumagillin and agents which potentiate angiogenesis inhibition such as heparin and sulfated cyclodextrin, particularly beta cyclodextrin tetradecasulfate.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates the inhibition of neovascularization stimulated by bFGF by treatment with fumagillin in accordance with the rat corneal micropocket assay, which is disclosed in detail in Example 4.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel methods for the treatment and/or prevention of angiogenesis-related diseases in mammals. The method, in its simplest form, comprises administering to the mammal an effective amount of fumagillin or a pharmaceutically acceptable salt thereof. The invention also provides certain pharmaceutical compositions comprising fumagillin or its salt, and an agent which potentiates angiogenesis inhibition such as heparin and sulfated cyclodextrins such as beta-cyclodextrin tetradecasulfate.

In accordance with the present invention, fumagillin has been found to be a strong inhibitor of angiogenesis in mammals. In treating angiogenesis-related diseases an effective amount of the free form, or a salt of fumagillin may be used. As the salt, inorganic salts such as alkali metal salt, e.g. sodium salt, potassium salt, alkaline-earth metal salt, e.g. calcium salt, and ammonium salt are preferred.

On the basis of its strong angiogenesis inhibitory activity, fumagillin and its salts are particularly useful for prophylaxis and treatment of diseases in the fields of ophthalmology, dermatology, pediatrics, surgery and cardiology.

Thus, fumagillin and its salts may be used for prophylaxis and/or treatment of neovascularization in diabetic retinopathy, retrolental fibroplasia, corneal graft neovascularization, neovascular glaucoma, ocular tumors, and trachoma; dermatological psoriasis and pyogenic granuloma; childrens hemangioma, angiofibroma and hemophiliac joints; and hypertrophic scars, wound granulation, vascular adhesions, rheumatoid arthritis, scleroderma and atherosclerotic plaque. Among these diseases, fumagillin and its salts are especially effective against diabetic retinopathy.

The use of fumagillin in accordance with the present invention has been found to be low in toxicity and is safely administered orally or parenterally to mammals (e.g. rat, rabbit, monkey, man) in forms of e.g. tablets, granules, capsules, injectable solutions, topical creams, and eye-drops.

To treat diabetic retinopathy, for example, fumagillin is administered orally in an amount of from about 1 mg/kg to 200 mg/kg per day, preferably in an amount of from about 2 mg/kg to 100 mg/kg per day. For oral administration, 5 mg to 100 mg of fumagillin or its salts may be formulated as a tablet or a capsule together with

a pharmaceutically acceptable carrier, diluent or other suitable vehicle.

Fumagillin may also be administered subcutaneously or intravenously in an amount of from about 0.1 mg/kg to 20 mg/kg per day to an adult in the form of a pharmaceutical acceptable composition. Preferably, it is administered in an amount of from about 0.2 mg/kg to 10 mg/kg per day. The preferred form of fumagillin for intravenous administration is as a sodium salt.

Fumagillin may also be administered topically. For example, to treat eye-related angiogenesis diseases, fumagillin, especially as a salt, may be administered in the form of eye-drops. One to a few drops per dose is administered to the eye with a frequency of 1 to about 4 times a day according to the patient's condition. Preferably, the eye-drops are prepared by dissolving a fumagillin salt in distilled water to make a concentration of 0.001 to 3% (w/v). The solution also preferably contains an isotonizing agent, a preservative, or a thickening agent and is adjusted to a pH of from about 5 to 9.

In accordance with another aspect of the present invention, it has been found that certain agents potentiate the angiogenesis inhibitory activity of fumagillin. Such agents include heparin and a group of compounds known as sulfated cyclodextrins.

Heparin, an alpha, beta glycosidically linked highly sulfated copolymer of uronic acid and glycosamine, has been used clinically as an anticoagulant for half a century. Despite its importance and widespread use, both the exact structure of heparin and the precise nature by which it acts in blood anticoagulation have not been discovered. Much of the difficulty in determining the structure of heparin results from the fact that it is not a homogeneous well-defined substance. Heparin is polydisperse with a molecular weight range from 5,000 to 40,000. Within a given chain, there are structural variations such as the varying degrees of sulfation, N-acetylation and C-5 epimerization in the uronic acid residue.

Cyclodextrins are natural cyclic compounds consisting of six (alpha), seven (beta) or eight (gamma) D-glucose units. It has a donut-shaped molecular structure which provides a cavity whereby clathrates may form with guest molecules of suitable size. The interior of the cavity consists largely of uniformly spaced bridging acetal oxygen atoms. One end of the cavity is edged with —CH_2OH Groups (one per glucose unit) and the other rim is similarly edged with secondary —CHOH Groups. The cavity contains water molecules hydrogen bonded to the interior oxygen atoms.

The alpha, beta, and gamma cyclodextrin sulfate salts are all usable as potentiating agents of fumagillin in accordance with the present invention. Beta-cyclodextrin salts are such as beta-cyclodextrin tetradecasulfate are preferred.

As discussed in more detail below, the angiogenesis inhibitory activity of fumagillin is markedly potentiated by sulfated polysaccharides such as heparin and beta cyclodextrin tetradecasulfate.

Thus, such agents may be used in conjunction with fumagillin in the prophylaxis or treatment of angiogenesis-related diseases. As the skilled artisan will appreciate, the relative amount of such potentiating agents to fumagillin may vary depending on a number of factors, including the patient's condition and administration route. In general, the ratio of potentiating agent to fumagillin by weight is between about 1:10 to 30:1, preferably from about 1:3 to 10:1.

The invention will be further illustrated with reference to the following examples which will aid in the understanding of the present invention, but which are not to be construed as a limitation thereof.

EXAMPLE 1

Shell-less Chorioallantoic Membrane Assay

The shell-less chorioallantoic membrane (CAM) assay was carried out by the method of Taylor and Folkman (S. Taylor and J. Folkman; Nature, 297, 307 (1982)) with a slight modification as follows: 3-days chick embryos were removed from their shells and cultured in plastic cups on hammocks of plastic wrap. The sodium salt of fumagillin 10 ug, along with acidic FGF 200 ng (bovine brain, R & D Systems, Inc.), and methylcellulose 30 ug (Fisher Scientific Co., 4000 centipoise) was placed on plastic disks (polypropylene, 6 mm in diameter). After the solution had dried, the disks were placed on the CAM of 10-day embryos. Three days later, inhibition of neovascular formation by fumagillin was observed under a stereoscope (X 20, SMZ-10, Nikon), and compared to the control disk containing acidic FGF 200 ng as a stimulant of angiogenesis and methylcellulose 30 ug without fumagillin. Fumagillin showed angiogenesis inhibitory activity by the CAM assay (Table 1).

TABLE 1

Angiogenesis inhibitory activity of fumagillin by CAM assay.	
Number of disk showing angiogenesis inhibition/number of disk tested	
Exp. 1	3/3
Exp. 2	2/6
Exp. 3	6/9

EXAMPLE 2

Shell-less Chorioallantoic Membrane Assay

The shell-less chorioallantoic membrane (CAM) assay was carried out by the method of Folkman et al. (R.Crum. S.Szabo and J.Folkman; Science. 230. 1375 (1985)) as follows: three-day chick embryos were removed from their shells to petri dishes (Falcon 1005) under sterile hood and cultured for further 3 days.

The sodium salt of fumagillin and/or sulfated polysaccharide (e.g. heparin, beta cyclodextrin tetradecasulfate) were dissolved to a 0.45% methylcellulose aqueous solution and aliquots of 10 ul were pipetted onto Teflon rods. After the solution had dried, the methylcellulose disks (about 2 mm in diameter) containing test compounds, thus prepared, were implanted on the CAM of 6-day embryos.

After cultures for 48–72 hours, formations of avasuclar zones around the disks were observed with a stereoscope.

Percent of avascular zones was calculated by counting the disks forming avascular zones per the total disks tested. In each group there were 8–16 embryos.

As shown in Table 2, Fumagillin showed strong angiogenesis inhibitory activity by the CAM assay. Angiogenesis inhibitory activity of fumagillin was markedly potentiated by sulfated polysaccharides: heparin and beta cyclodextrin tetradecasulfate. Hydrocortisone does not potentiate and may suppress the effects of fumagillin.

TABLE 2

Angiogenesis inhibitory activity of fumagillin and potentiation by sulfated polysaccharides.

compound tested/disk		percent of avascular zones
fumagillin sodium salt	50 ug	57%
	40 ug	72%
	30 ug	62%
	20 ug	66%
	10 ug	75%
fumagillin sodium salt + hydrocortisone	10 ug 60 ug	40%
fumagillin sodium salt + heparin	10 ug 50 ug	100%
fumagillin sodium salt	5 ug	40%
fumagillin sodium salt + beta cyclodextrin tetradecasulfate	5 ug 25 ug	70%

EXAMPLE 3

Mouse dorsal air sac assay

The angiogenesis assay by mouse dorsal air sac method (MDA) was carried out by the method below which is a modification of the original rat dorsal air sac assay developed by Folkman et al. (Folkman, J. et al.; J.Exp.Med.,133, 275 (9171)). Specifically, millipore chambers, equipped with a millipore filter with a pore size of 0.45 um (Millipore Corp.), were filled with 5×10^6 sarcoma 180 cells in 0.15 ml of saline. The control contained the same volume of saline.

Under Nembutal anesthesia, each mouse received both the control and the tumor cell-containing chambers in their dorsal air sac produced by the method mentioned above. Fumagillin was subcutaneously administered to the mice for 3 days after the day of operation as a solution or suspension in 0.5% arabic gum dissolved in saline. Unless otherwise specified, fumagillin was given at a dose of 100 mg (10 ml)/kg body weight. Four days later, the mice were given an intra arterial injection of carmine-gelatin solution under Nembutal anesthesia, and cooled on ice for about 2 hours to let the gelatin-containing blood form gels according to the method of Kimura (Kimura, M. et al.; J. Pharmacobiol. Dyn., 9, 442 (1986)). The skin was incised widely over the chambers and inner surface of the skin was exposed. The mouse fascia was observed under a stereoscope (X20; SMZ-10, Nikon). The anti-angiogenic activity of the compounds was evaluated by determining the extent of the tumor-induced vascular formation in mice administered the vehicle or the test compounds.

Tumor-induced vasculature exhibiting both coiling vessels and an increase of the vascular net was defined as positive angiogenesis.

As the results in Table 3 show, fumagillin effectively inhibited the tumor-induced vascular formation.

TABLE 3

Inhibition of angiogenesis induced by tumor cells by fumagillin

	tumor-induced angiogenesis positive mice/total mice tested)
vehicle (arabic gum-saline)	5/6
fumagillin treated (100 mg/kg, s.c.)	0/5

EXAMPLE 4

Rat corneal micropocket assay

The rat corneal micropocket assay was carried out essentially by the method of Gimbrone et al. (Gimbrone, M.A. Jr. et al., J. Natl. Cancer Inst. 52, 413(1974)). Corneal vascularization was induced in adult male rats of the Sprague-Dawley strain (Charles River, Japan) by implanting 500 ng of basic FGF (bovine, R & D Systems, Inc.) impregnated in EVA (ethylene-vinyl acetate copolymer) pellets in each cornea (n=5). On the same day, fumagillin (5 ug/pellet) was also impregnated in EVA and implanted into the same cornea between the FGF pellet and the limbus. Control rats received an implantation of the FGF pellet and the EVA pellet without fumagillin. Neovascular formation was observed under a stereoscope (X20, SMZ-10, Nikon). The extent of vascularization was graded from — to +++ (—, without neovascularization; +, positive neovascularization not yet reaching the FGF pellet; ++, positive neovascularization reaching the FGF pellet; +++, positive neovascularization surrounding the FGF pellet).

As shown in FIG. 1, neovascularization stimulated by bFGF was effectively inhibited by fumagillin.

EXAMPLE 5

In a similar experiment approximately 10 to 16 chick embryos (age 6 days) were used to assay each concentration of fumagillin substantially in accordance with the method described in Example 2. The fumagillin was applied in a 10 ul pellet to the chorioallantoic membrane with or without cyclodextrin. Forty-eight hours later, the percent of embryos with an avascular zone was recorded. The results are shown in Table 4. Unless otherwise indicated, the avascular zones are 1+, e.g., at least 2 mm in diameter. An avascular zone of 2+ equals 4 mm or greater in diameter, indicating very high activity. For example, 5 ug of fumagillin produced 33% avascular zones. In contrast, 5 ug of fumagilin plus 25 ug of beta-cyclodextrin tetradecasulfate produced 100% avascular zones (the majority of which are 2+). Cyclodextrin (25 ug) alone had no detectable effect. Heparin also potentiates fumagillin, but it is not as potent as beta-cyclodextrin in this respect (data not shown).

TABLE 4

Fumagillin ug/10 ul	B Cyclodextrin tetradecasulfate ug/10 ul	Percent Avascular Zones
50	0	57%
40	0	72%
30	0	62%
20	0	75%
10	0	75%
5	0	33%
2.5	0	40%
1	0	0%
5	25	100% (2+)
2.5	25	57%
1	25	33%
Fumagillin	Heparin	
10	50	100%

B cyclodextrin alone = 0% avascular zones.
Heparin alone = 0% avascular zones.

EXAMPLE 6

A pharmaceutical preparation for use as eye-drops was prepared as follows:

fumagillin sodium salt	1 g
boric acid	16 g
sodium borate	7 g
p-hydroxy-benzoic acid methylester	0.25 g
p-hydroxy-benzoic acid propionylester	0.15 g

Sterile distilled water was added to total 1 liter. After sterilization by filtration, the solution was used as eye-drops.

EXAMPLE 7

A pharmaceutical preparation for use as eye-drops was prepared as follows:

fumagillin sodium salt	5 g
p-hydroxy-benzoic acid methylester	0.25 g
p-hydroxy-benzoic acid propionylester	0.15 g
dibasic sodium phosphate	4. g
sodium chloride	8.5 g

Sterile distilled water was added to total 1 liter. The pH was adjusted to a pH 7.5. After sterilization by filtration, the solution was used as eye-drops.

What is claimed is:

1. A method of inhibiting angiogenesis in a mammal, comprising administering an amount of fumagillin effective for the inhibition of angiogenesis or a pharmaceutically acceptable salt thereof to the mammal.

2. A method of preventing angiogenesis in a mammal, comprising administering an amount of fumagillin effective for the prevention of angiogenesis or a pharmaceutically acceptable salt thereof to the mammal.

3. The method according to claim 1 or 2, wherein the angiogenesis is caused by abnormally stimulated neovascularization.

4. The method according to claim 3, wherein the abnormally stimulated neovascularization is diabetic retinopathy.

5. The method according to claim 3, wherein the abnormally stimulated neovascularization is trachoma.

6. The method according to claim 1 or 2, wherein fumagillin is administered orally in amount of 0.1 mg/kg to 200 mg/kg per day.

7. The method according to claim 1 or 2, wherein the fumagillin is administered intravenously in an amount of 0.1 mg/kg to 20 mg/kg per day.

8. The method according to claim 1 or 2, wherein an aqueous solution of fumagillin sodium salt at a concentration from 0.001% to 3% (w/v) is administered in the form of eye-drops.

9. The method of claim 1 or 2, further comprising administering in conjunction with fumagillin or a salt thereof, an effective amount of a potentiator of said fumagillin selected from heparin and a sulfated cyclodextrin, the ratio of potentiator to fumagillin by weight being between about 1:10 to 30:1.

10. The method of claim 9, wherein the sulfated cyclodextrin is beta cyclodextrin tetradecasulfate.

11. A pharmaceutical composition for the treatment of angiogenesis in mammals comprising fumagillin or a pharmaceutically acceptable salt thereof and a potentiator of said fumagillin selected from heparin and a sulfated cyclodextrin, the ratio o potentiator to fumagillin by weight being between about 1:10 to 30:1.

12. The pharmaceutical composition of claim 11, wherein the sulfated cyclodextrin is beta cyclodextrin tetradecasulfate.

13. The pharmaceutical composition of claim 11 or 12, further comprising a pharmaceutically acceptable carrier.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,135,919

DATED : August 4, 1992

INVENTOR(S) : Folkman, et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On column 8, line 31, please change "ratio o" to --ratio of--.

Signed and Sealed this

Sixteenth Day of November, 1993

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks